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Introduction

Breast cancer remains the major cancer (excluding skin cancer) among women in the United States with more than 175,000 new cases anticipated in 1999. The requirement of estrogens for normal breast development is well documented (1). However, estrogens also have been linked to breast cancer, presumably through their ability to stimulate cell proliferation (2) and inhibition of estrogen action therefore has been a primary objective in the treatment, and more recently the prevention of, breast cancer. For many years, estrogen effects were thought to be mediated by a unique, high affinity intracellular receptor protein, the estrogen receptor (ER), that is a member of a superfamily of transcription factors (3,4). The basic mechanisms of ER activity have been ascertained. Hormone binding to ER results in receptor homodimerization and binding to specific enhancer DNA elements located in the promoter regions of target genes (5,6). This process, which is accompanied by increases in ER phosphorylation (7-12), enables "activated" receptors to regulate the transcription of hormone-responsive target genes and the resulting changes in mRNA and protein synthesis are ultimately responsible for alterations in cellular function. The structural features of the estrogen receptor (ER α) responsible for hormone binding, dimerization, DNA binding and transcriptional activation have been identified (3,13-16) and these studies have provided the basis of our understanding of the molecular mechanisms by which estrogens regulate the growth and differentiation of mammary tissues.

Clearly, the transcriptional activity of the ER is regulated by estrogens, such as 17 β -estradiol (E₂). However, the ER α also can be activated in the absence of exogenous ligand by agents that stimulate intracellular signal transduction cascades (EGF, IGF-1, heregulin, dopamine, TPA and cAMP) (7,17-23) or inhibit protein phosphatases (okadaic acid) (19). Furthermore, cyclin D1, independent of cyclin-dependent kinases, also can activate the ER in the absence of estrogen (24). The ER α knock-out mouse model confirms that ER α is required for some but not all *in vivo* EGF effects and established the importance of ligand-independent activation of ER to physiological events (25). Most of these ligand-independent activation pathways (with the exception of cyclin D1) increase receptor phosphorylation (7,12,23,26,27) and mutation of the only known ligand-independent (EGF) phosphorylation site (serine¹¹⁸) to an alanine residue abolishes EGF activation of the ER (28), suggesting that phosphorylation may play an important role in these activation pathways. However, this point mutant does not block cAMP-mediated gene expression and different domains are required to respond to EGF and cAMP signaling pathways (29), suggesting that multiple mechanisms must exist to enable ER to activate target gene expression in response to diverse regulatory events.

In 1996, a new member of the nuclear receptor superfamily was cloned from a prostate cDNA library (30). When the resulting cDNA was sequenced and expressed, it became apparent that a novel estrogen receptor had been identified. This new member of the nuclear receptor superfamily was named ER β , and the original estrogen receptor was renamed ER α . The ER β binds to estradiol with an affinity (K_d 0.4 nM) similar to ER α and binds to the same DNA response element as ER α (30-32). Thus, it is reasonable to predict that ER β regulates the expression of at least a subset of ER α target genes. However, the relatively undeveloped mammary glands in the ER α knock-out mouse indicate that ER β is not equivalent to ER α (33). The reasons for this are unclear, but could be related to differential expression and/or differences in the ability of α and β receptors to activate target gene expression. Mouse, rat and human ER β s are approximately 65 amino acids smaller than their corresponding α -receptors, and the

A/B, D and F regions (**Figure 1**) are poorly conserved (30,32,34). Furthermore, the ligand binding domains (region E) of ER α and ER β are only ~55% identical and rat ER α and ER β receptors do not bind equally well to all ligands (31). The expression patterns of ER α and ER β mRNAs are different but overlapping (31) suggesting that the genes for ER α and ER β are independently regulated. However, both ER α and ER β mRNA have been detected in human mammary gland, breast tumors and several human breast cancer cell lines (35,36). Taken together, these data suggest that ER β is likely to play a role in mediating estrogen action in mammary gland, but that this receptor is unlikely to be functionally equivalent to ER α .

The identification of a second estrogen receptor raised a number of important biological questions such as, what is the expression of ER β , relative to ER α , in normal and malignant mammary tissue? However, knowledge regarding the expression of ER β in mammary gland will be of limited value without detailed information on the transcriptional activity of ER β . Do ER α and ER β respond similarly to ligand-independent pathways? Are currently used antiestrogens equally effective antagonists of ER α and ER β ? Do ER α and ER β activate the same target genes to a comparable extent? Studies of this nature will provide the information necessary to determine whether resources are required to develop new strategies to more effectively and/or selectively block estrogen and estrogen receptor (α and β) action. Indeed, if ER α and ER β are not functionally equivalent, experiments, such as those reported below, will provide a solid foundation upon which new strategies to regulate ER α and ER β biological activity can be developed. Moreover, comparing and contrasting the structure/function relationships of ER α and ER β with respect to activation by ligand-independent pathways represents a novel approach to study mechanistic questions relating to activation of gene expression in the absence of estrogens.

Body

A new member of the nuclear receptor superfamily, ER β , has been identified that binds to estrogens with high affinity, and binds to the same DNA response elements as the classical estrogen receptor, ER α . Both of these ligand-regulatable transcription factors possess a well-defined, centrally located, DNA binding domain and carboxy-terminal domain, which contains a ligand-dependent activation function (AF-2); however the amino terminus which possesses a second activation function (AF-1) is poorly conserved. Thus, it is highly likely that the biological activity of ER β will differ from that of ER α . This hypothesis is being tested in the following two technical objectives:

1. To determine if estrogen-independent signaling pathways can stimulate ER β transcriptional activity.
2. To determine what regions of ER β contribute to its estrogen-independent transcriptional activity and to compare these regions to known ER α activation functions to characterize the structural features of these receptors that contribute to their respective biological properties.

The first ER β cDNA published was isolated from a rat cDNA library and encoded a protein of 485 amino acids (30). Based on this report other investigators cloned the cDNAs for human and mouse ER β , which encoded proteins of 478 and 485 amino acids, respectively (32,34). In

order to begin our studies of the ability of cell signaling pathways to activate ER β -dependent transcriptional activity, it was necessary to construct expression vectors for ER α and ER β such that each cDNA would be expressed from the same vector backbone. This is important to ensure that any differences in gene expression observed in subsequent studies could be attributed to differences in the receptors themselves and not to alterations in the relative expression of these proteins. Our ER β cDNA was obtained in the vector, pCMV₅. Therefore, the ER α cDNA was isolated from the pJ3 Ω vector (37), and subcloned into the *Eco*RI site of pCMV₅. Upon accomplishing this task, the transcriptional activity of ER α and ER β was characterized in a transient transfection system to assess their respective abilities to regulate synthetic target gene expression in an estrogen-dependent manner. In order to perform these experiments, expression vectors for either ER α or ER β were cotransfected into HeLa cells in the presence of a synthetic target gene, ERE-E1b-Luc, which consists of an estrogen response element (ERE) upstream of the E1b TATA box and the luciferase gene. When 10 ng of the respective expression vectors were used, it was noted that in estrogen-treated cells expressing ER α that the transcriptional activity was approximately 5-fold higher than in cells expressing ER β (**Figure 2**). However, in titration experiments, increasing the amount of ER β expression vector resulted in higher luciferase gene expression, suggesting the possibility that the relatively low ER β transcriptional activity was due to poor expression of ER β protein from the vector. Because the original ER β cDNA had a very long (423 nucleotides) 5'-untranslated region (UTR), the possibility that this region of the cDNA was affecting the relative expression of ER β was examined. An ER β expression vector was constructed in which the first 399 nucleotides were removed by restriction digestion with *Acc*I and subcloning back into pCMV₅, and the resulting ER β _s expression vector was again tested in transient transfection assays. This construct produced a receptor that was more active than the original ER β , such that ER α was only 2.9-fold more active than ER β _s (**Figure 3**). As a control, the parent vector, pCMV₅, was also transfected into cells with the ERE-E1b-Luc reporter gene, and as expected, no significant activity was observed, indicating that target gene expression was ER-dependent. In a more detailed experiment, increasing levels of expression vector for the original ER β expression vector (hereafter referred to as ER β _L) and the modified expression vector (ER β _s) were titrated into cells along with the target gene, and luciferase activity was assessed. This experiment confirmed that ER β _s stimulated more gene expression when low levels of expression vector (e.g. 10 ng) were used (**Figure 4**). However, when higher levels of expression vector were employed, the ER β _L construct produced more luciferase activity. This suggested that the putative 5'-UTR region might be controlling the expression of ER β protein in HeLa cells, and that alterations in receptor levels were contributing to the relative differences obtained in the *trans*-activation assays.

In order to determine whether the relative differences in target gene expression observed in cells transfected with the two ER β expression vectors reflected variations in the relative expression of ER β _s and ER β _L protein, both hormone binding and Western blot analyses were performed. Increasing amounts of expression vectors for ER β _L, ER β _s, or pCMV₅ were transfected into HeLa cells, and 24 hours thereafter, cell extracts were prepared. Binding assays were performed with [³H]estradiol in the presence and absence of a 100-fold excess of unlabelled 17 β -estradiol. Separation of free and bound steroid was achieved with hydroxyapatite (38). This analysis revealed that the ER β _s form of receptor was expressed, on average, at approximately 1.4 times the level of the original ER β construct (**Figure 5**), supporting the hypothesis that the

higher apparent transcriptional activity of ER β s may simply reflect its relatively higher level of expression. No binding was detected in cells transfected with the pCMV₅ parent vector alone. In order to confirm these observations, Western blot analysis was performed with extracts prepared from cells transfected with pCMV₅, the original ER β _L or the truncated ER β _S expression vectors. Several commercial manufacturers have developed commercially available antibodies against ER β since submission of this proposal. We evaluated antibodies from Affinity Bioreagents and Upstate Biotechnology, and in pilot experiments found that the Affinity Bioreagents antibody was able to recognize ER β with superior specificity and affinity. This antibody was used in the Western blot analysis presented here, and will be used in subsequent years to examine ER β phosphorylation. The availability of this antibody has made it unnecessary to generate an expression vector for Flag-epitope-tagged ER β at this time. As shown in **Figure 6**, the expression level of ER β _S was approximately 10-fold greater than that achieved for the original clone (ER β _L). More importantly however, was the observation that the two forms of ER β migrated with different mobilities. The original ER β construct appeared to encode a protein of ~60 kiloDaltons, while the expression vector in which the putative 5'-UTR had been deleted encoded a protein of ~56 kiloDaltons. This prompted us to sequence the putative 5'-UTR and upon doing so, an additional nucleotide (a C residue at nucleotide position 324 relative to the originally published sequence, reference (30)) that had not been reported in the original publication or database deposit of the ER β sequence was discovered. Inclusion of this additional nucleotide extended the open reading frame for ER β by an additional 45 amino acids. Within the last year, several other laboratories also have reached the same conclusion about the size of the full-length form of ER β (39,40). Therefore, there are two forms of ER β that we and others have investigated to date. The full-length form is 530 amino acids in length, while the truncated version is 485 amino acids long. Although we plan to use the full-length form for all of our subsequently planned studies, some of our early ER β functional analyses have been performed with expression vectors for the short form of ER β .

We have begun our analyses of the ability of ER β to be activated by ligand-independent signaling pathways. For these studies, we have compared the activity achieved in cells transfected with ER β (both long and short forms) in comparison to experiments that were performed in parallel examining the activity of ER α . We began by examining the receptor activity in cells that were treated with forskolin and isobutylmethylxanthine (IBMX). Forskolin is an activator of adenylyl cyclase and IBMX is a phosphodiesterase inhibitor, and treatment of cells with these compounds results in an increase in intracellular cAMP production. In transient transfection assays, 10 μ M forskolin and 100 μ M IBMX stimulated the activity of ER β by 2.4-fold while the activity of the short form of ER β (ER β _S) was stimulated only 1.9-fold (**Figure 7**). In comparison, the activity of ER α was stimulated by 2.4-fold. Thus, intracellular cAMP signaling pathways have the potential to activate the transcriptional activity of ER α and both forms of ER β . Because these receptors have regions of high (DNA and ligand binding domains) and low identity (amino-terminal domains), it will be possible in the upcoming year to examine the structural regions that enable both receptors to respond to this signaling pathway. Other signal transduction pathways outlined in the original proposal will be examined in year two [e.g. EGF, IGF, okadaic acid (an inhibitor of protein phosphatase 2A), phorbol-12-myristate-13-acetate (an activator of protein kinase C pathways) and cyclin D₁].

The ability of antiestrogens to block cAMP-dependent activation of ER β -dependent transcription was also assessed. Both the mixed antiestrogen, 4-hydroxytamoxifen (4HT) and the pure antiestrogen, ICI 164,384, were able to inhibit activation by this ligand-independent activation pathway (**Figure 8**). This contrasts with the inability of 4HT to block cAMP-dependent activation of ER α transcriptional activity (41), and the structural differences between the two receptor isotypes will be examined in the coming year to understand the mechanistic basis for these contrasting responses to antiestrogen treatment.

With respect to the second technical objective, work in the first year of this project has focussed on constructing vectors that will enable us to examine the structural features that enable ER α and ER β to respond in equal or dissimilar ways to various estrogen-independent signals. Expression vectors for chimeric proteins have been generated in which the amino-terminal domains of ER α and ER β (individually), have been fused C-terminal to the heterologous Gal4 DNA binding domain (DBD). These constructs isolate the A/B region which encompasses the AF-1 domain from the remainder of the respective receptor and will facilitate an examination of their transcriptional activity in response to ligand-independent signaling pathways. To assess the transcriptional activity of the A/B regions of ER α and ER β in unstimulated cells, the respective expression vectors were transiently transfected into HeLa cells along with a synthetic target gene consisting of 5 copies of the response element to which the Gal4 DBD binds. This experiment demonstrated that the A/B domain of ER α has significantly more transcriptional activity than the Gal4 DBD alone (**Figure 9**). In contrast, the A/B domain of ER β has weak activity in comparison to ER α , although it is distinguishable from that observed for the GAL4 DBD alone. These expression vectors will be used in the following year to examine the ability of signaling pathways to alter the activity of this isolated activation function. Expression vectors for ER α and ER β deletion mutants in which the A/B domains have been deleted, or in which their E/F regions (lacking the DNA binding domain) will be fused C-terminal to the Gal4 DBD are currently being prepared. Collectively, these constructs will enable us to compare and contrast the ability of various estrogen-independent signaling pathways to activate a) the full-length receptor, b) the A/B region (AF1), c) the DNA and ligand binding domain and d) the ligand binding domain alone (AF2).

Finally, we have generated chimeras of ER α and ER β in which the A/B region of the ER α receptor was substituted for the A/B region of ER β to create a ER $\alpha\beta$ chimera and *vice versa*. These constructs have been tested for their ability to be activated by 17 β -estradiol (**Figure 10**) and as expected, we have observed that the ER α receptor is more active than ER β or either of the two chimeras (ER $\alpha\beta$ or ER $\beta\alpha$). Thus, we have prepared chimeras that will enable us to examine in the context of a full-length receptor whether estrogen-independent signals require the amino and carboxy-termini of the same receptor subtype, or whether the two receptor forms can substitute for one another.

Key Research Accomplishments

1. ER α is more active than ER β _S. Furthermore, ER β _S is more active than ER β _L in response to 17 β -estradiol stimulation for low amounts of transfected expression plasmids, whereas ER β _L is more active than ER β _S when higher levels of expression plasmid are transfected into cells.

2. ER β_S is expressed at a higher level than ER β_L for equal amounts of transfected expression vectors as indicated by hormone binding (≤ 2 -fold) and Western blot analyses (> 10 -fold).
3. Forskolin/IBMX activated ER α and ER β_L approximately 2.4-fold whereas ER β_S was activated even less than ER β_L .
4. The mixed antiestrogen, 4-hydroxytamoxifen, and the pure antiestrogen, ICI 164,384, inhibited the ability of ER β to activate target gene expression in response to estrogen and cAMP signaling pathways.
5. Expression vectors for chimeras of the A/B regions of ER α and ER β with the GAL4 DNA binding domain have been generated and characterized. The AF-1 activity of ER α is greater than the AF-1 activity of ER β .
6. Expression vectors for chimeras of the N-termini and C-termini of ER α and ER β have been constructed and characterized.

Reportable Outcomes

A portion of the work outlined in this progress report was presented at the 81st Annual Meeting of The Endocrine Society (San Diego, CA; June 12-15, 1999) in poster form. The abstract (see Appendix) was entitled "Activation of estrogen receptor- α and estrogen receptor- β by ligand-dependent and ligand-independent pathways".

Conclusions

The originally published amino acid sequence of ER β represents an amino-terminally truncated form, which lacks the first 45 amino acids of this receptor isotype. In transient transfection assays, ER α is clearly more active than the long and short forms of ER β . The potentially large differences in ER β_S and ER β_L expression levels indicate that their relative expression levels must be taken into account when considering transactivation activity. Furthermore, the AF-1 activity of ER α exceeds that of ER β and this likely contributes to the relative differences in transcriptional activity observed for these two receptor isotypes.

Both isotypes of estrogen receptor (α and β) can be activated in the absence of exogenous estrogens. In cells treated with forskolin and IBMX, ER α and both forms of ER β (short and long) were activated by a cAMP signaling pathway. This indicates that there is sufficient homology between these two receptor isotypes to mediate activation of gene expression by this signaling pathway. However, the responses of these receptors to inhibition of cAMP activation by 4-hydroxytamoxifen are distinct. This mixed antiestrogen inhibits cAMP-stimulated, ER β -dependent transcriptional activity, while 4-hydroxytamoxifen further activates ER α stimulated by cAMP signaling pathways. Thus, there are differences between these two receptor isotypes with respect to their regulation by antiestrogens. Expression vectors for various ER deletion mutants have been constructed which will enable us in the upcoming year to begin to examine which region(s) of these receptors is responsible for the differences in response.

As anticipated, the experiments performed to date have provided information on the transcriptional activity of ER β relative to ER α , as well as the ability of ER β to respond to an alternative signaling pathway, induced by elevated intracellular cAMP, in the absence of estrogens. Using the expression vectors for mutant forms of ER α and ER β constructed this year, information on the structural features of the two receptors that are critical for responsiveness to ligand-independent signaling pathways should be obtained. Taken together, this information will increase our understanding of the molecular mechanisms by which ER α and ER β respond to cross-talk within a cell. It also will provide a framework for critical evaluation of whether it is possible to selectively regulate ER α and ER β transcriptional activity.

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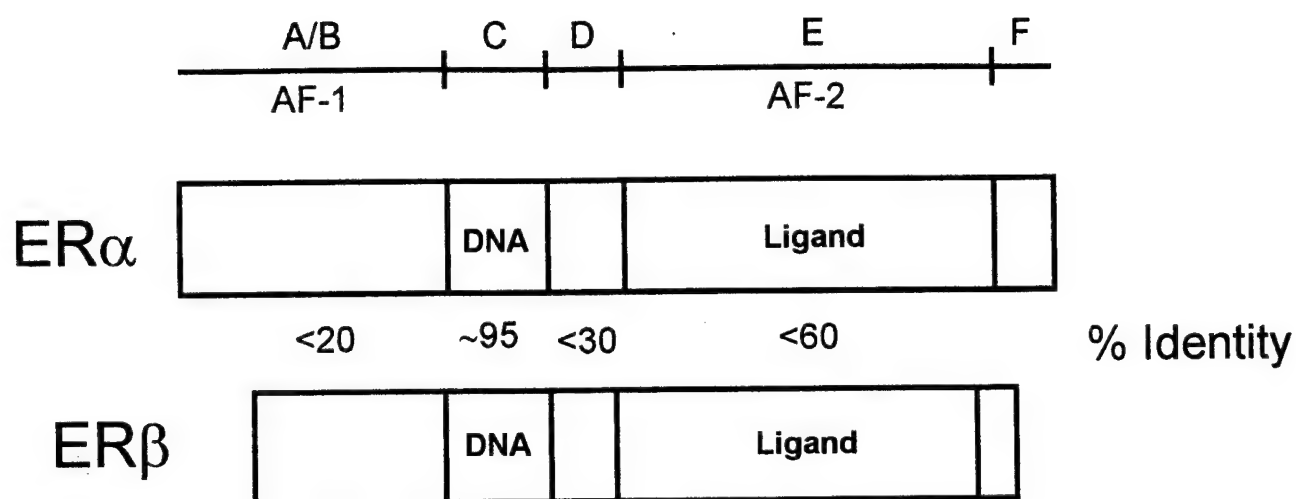


Figure 1: Structures of ER α and ER β . The *top panel* represents the location of various regions of estrogen receptor (A to F) and its activation functions (AF-1 and AF-2). The *bottom panel* represents the comparative structure of ER α and ER β . Values given between the two receptor forms represent approximate homologies in their respective amino acid sequences.

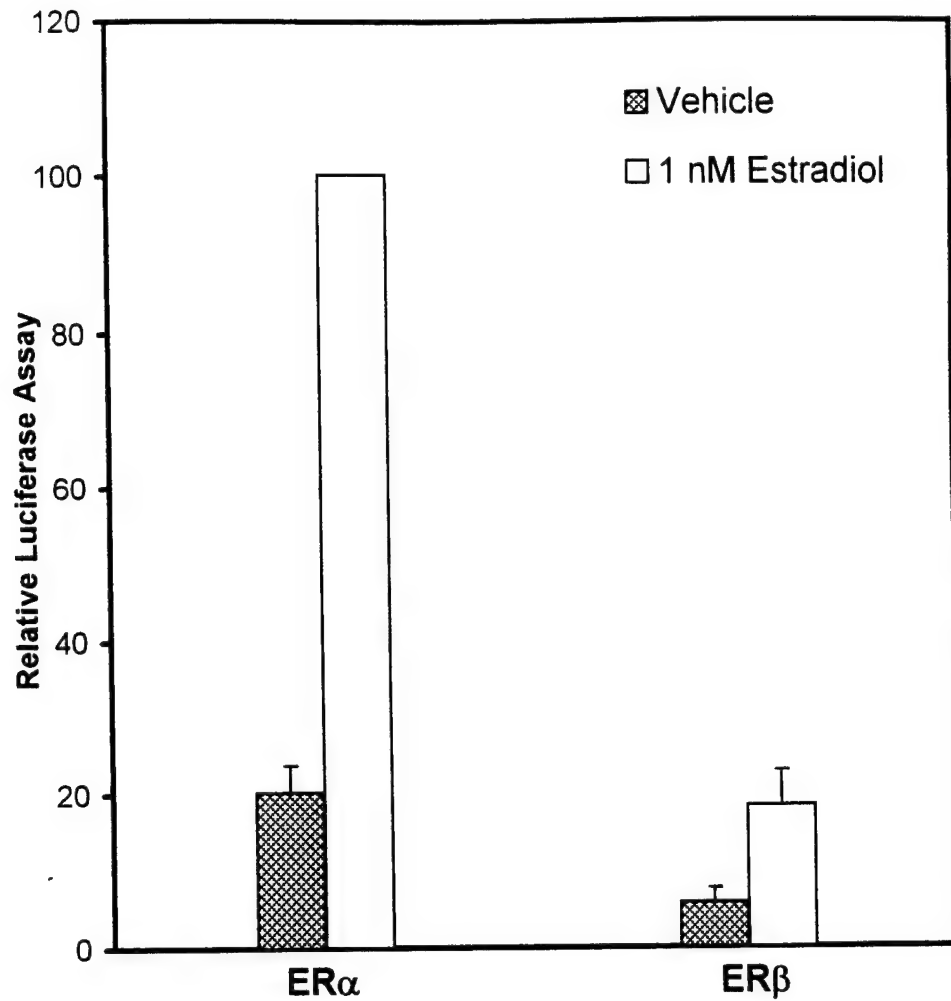


Figure 2: ERα is More Active than ERβ in Response to Stimulation with 17β-estradiol. HeLa cells were co-transfected with 1 μg ERE-E1b-Luc reporter plasmid and either 10 ng pCMV₅-ERα (ERα) or 10 ng pCMV₅-ERβ (ERβ). Luciferase measurements were corrected for protein values and standardized to ERα activity stimulated by 1 nM estradiol. Transfections were performed in duplicate and values represent the mean ± SEM of three experiments.

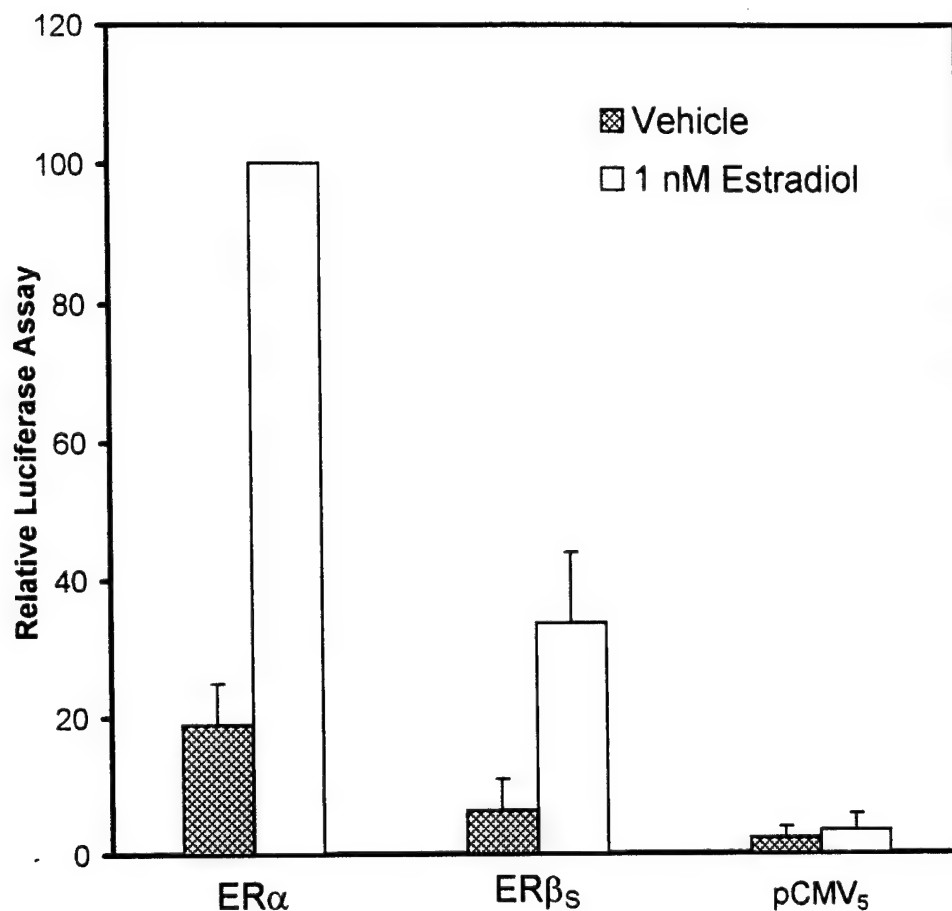


Figure 3: ERα is More Active than ERβ-short in Response to Stimulation with 17β-estradiol. HeLa cells were co-transfected with 1 μg ERE-E1b-Luc reporter plasmid and either 10 ng pCMV₅-ERα (ERα) or 10 ng pCMV₅-ERβ-short (ERβ_s). Luciferase measurements were corrected for protein values and standardized to ERα activity stimulated by 1 nM estradiol. Transfections were performed in duplicate and values represent the mean ± SEM of three experiments.

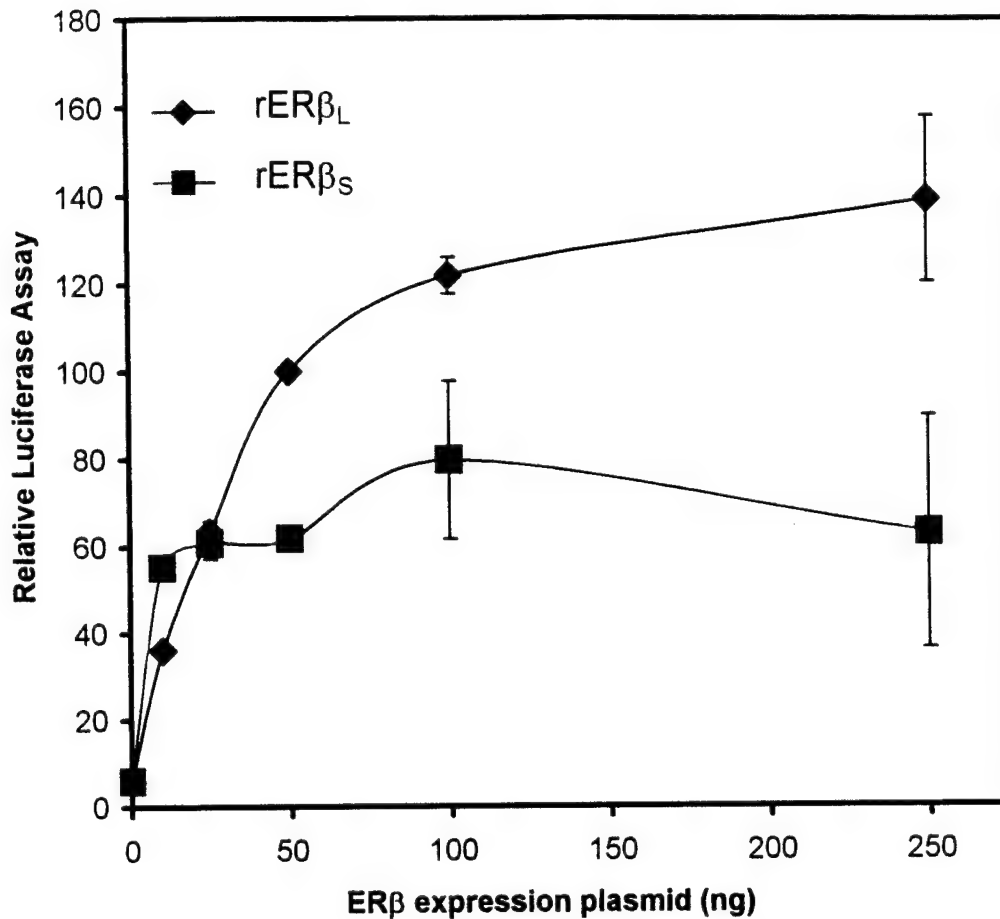


Figure 4: Comparison of ERβ-long and ERβ-short Transcriptional Activity. HeLa cells were transfected with 1 μg ERE-E1b-Luc, 100 ng CMVβgal, and the indicated amounts (*x-axis*) of pCMV₅-ERβ_L or pCMV₅-ERβ_S expression plasmids. Luciferase measurements, normalized to CMVβgal activity, are the results of stimulation with 1 nM estradiol. Transfections were performed in duplicate and values were standardized relative to values obtained for 50 ng of ERβ_L. Values represent the mean ± SEM of four experiments.

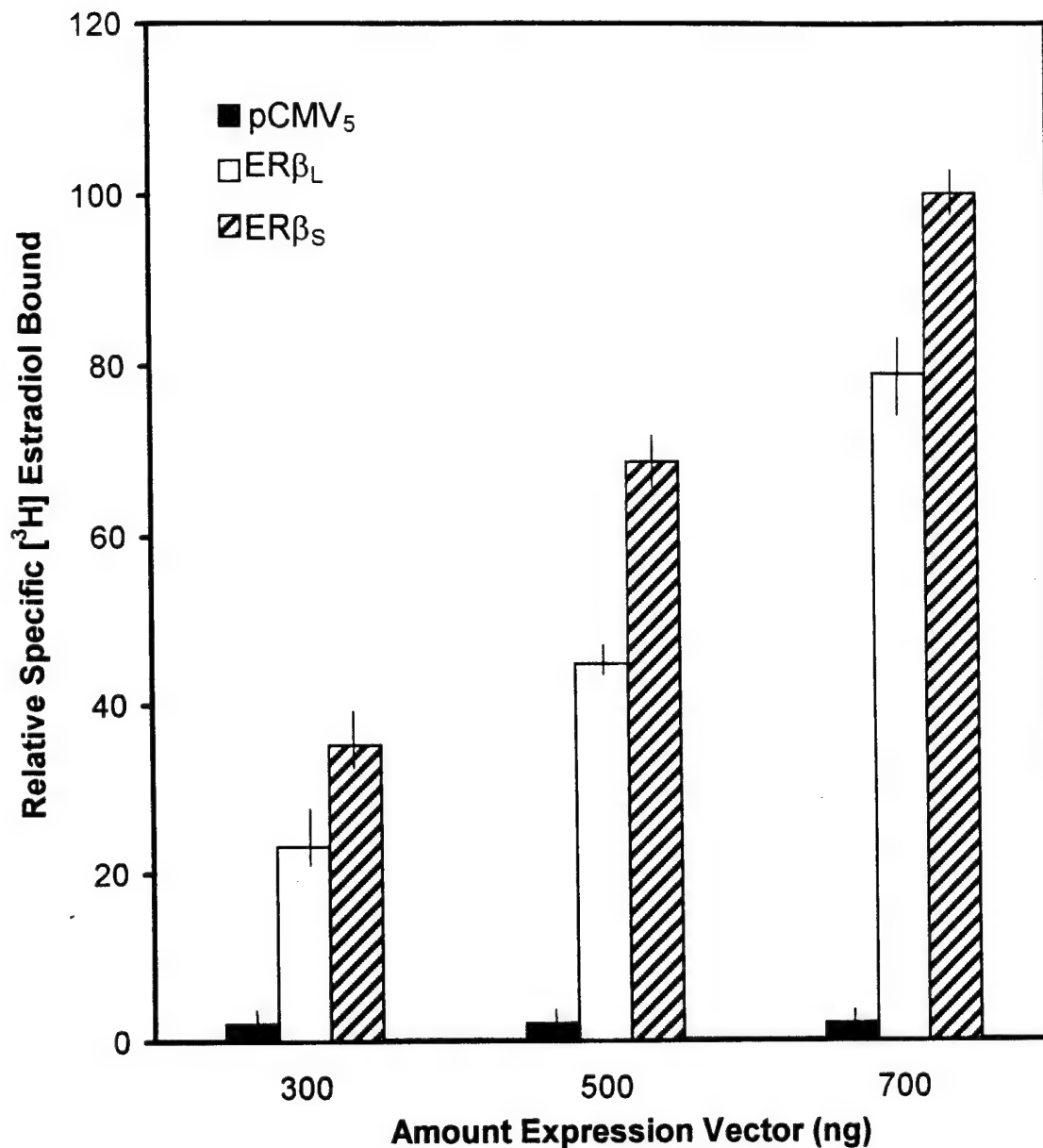


Figure 5: Relative Expression of ERβ-short and ERβ-long Determined by *In Vitro* Hormone Binding Analyses. HeLa cells were transfected with CMVβgal and the indicated amounts of either pCMV₅-ERβ_S or pCMV₅-ERβ_L expression plasmids, or the parent vector (pCMV₅). Binding assays were performed in duplicate and values represent the mean and range of relative specific counts bound, normalized to CMVβgal activities for two experiments.

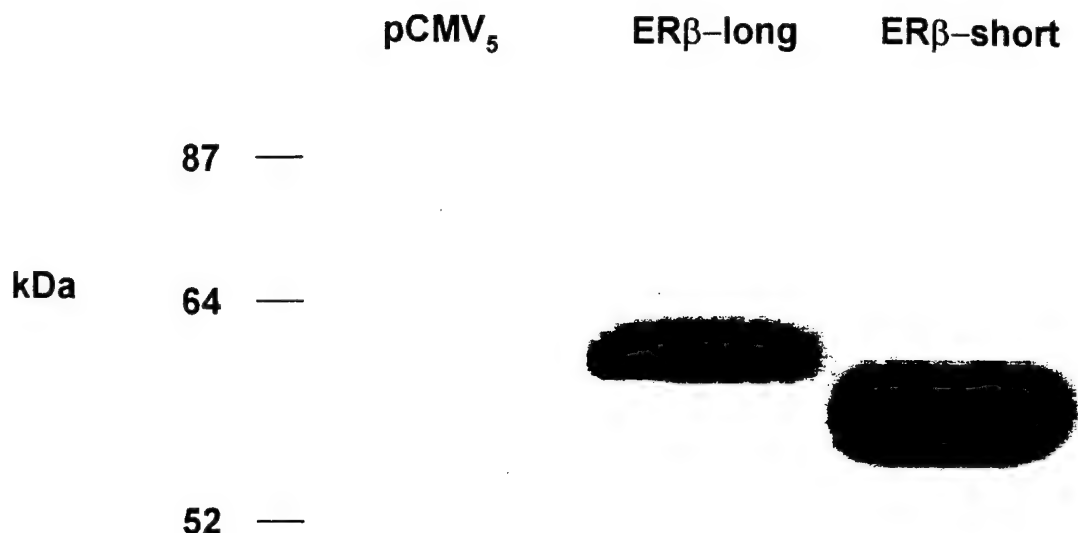


Figure 6: Relative Expression of ERβ-short and ERβ-long determined by Western Analyses. HeLa cells were transfected via adenovirus with pCMV₅-ERβ_S, pCMV₅-ERβ_L, or the parent vector and cells harvested twenty four hours after transfection. Cells were lysed in ER extraction buffer, protein concentrations determined by BioRad reagent, and an equal amount (25 μg) of protein was resolved by 7.5% SDS-PAGE. The resulting gel was blotted onto nitrocellulose membrane and an anti-ERβ antibody was employed for detection of the ER. Molecular size standards are shown to the *left* of the figure.

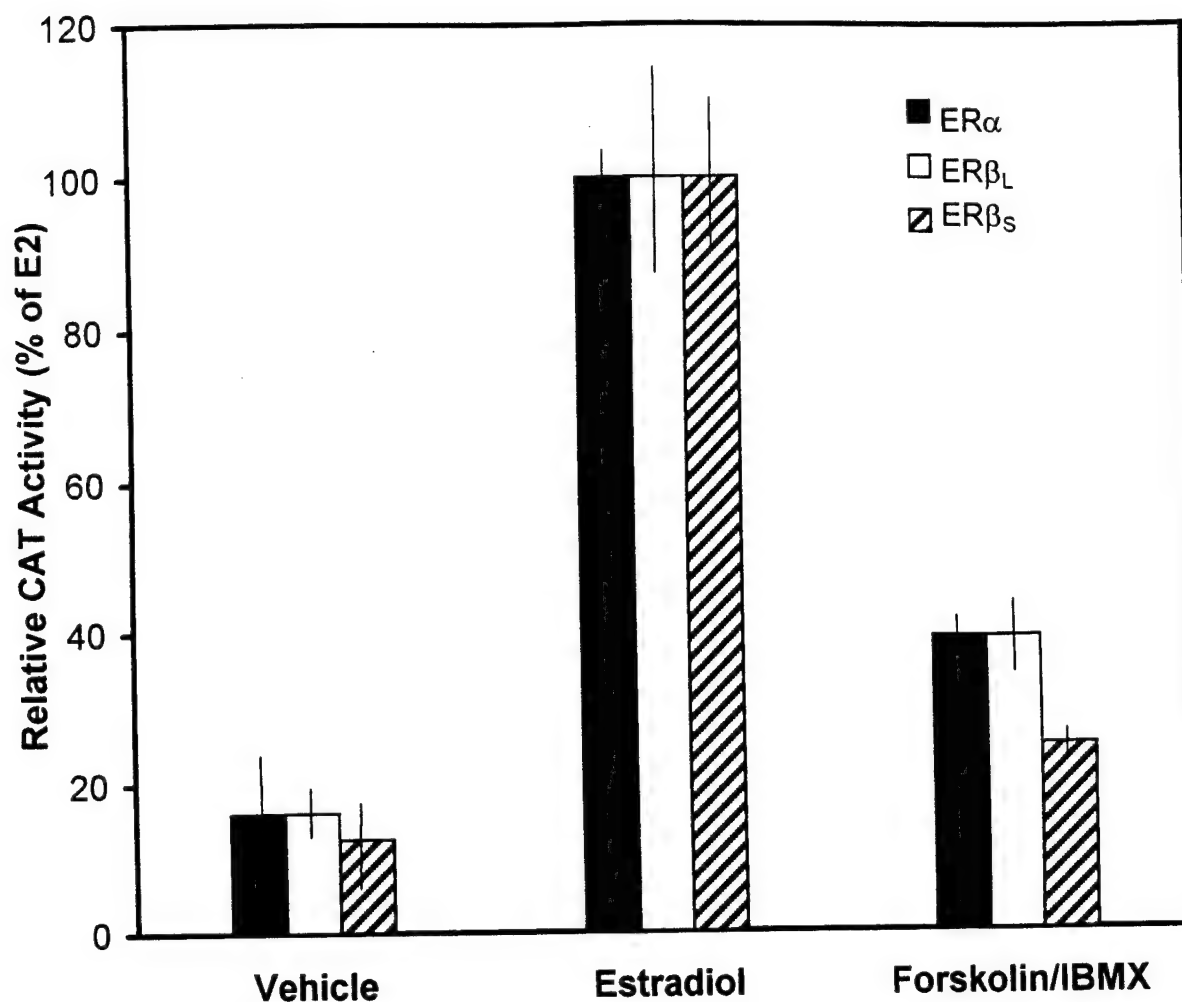


Figure 7: Forskolin/IBMX Activates ERα, ERβ-long and ERβ-short. HeLa cells were transfected with an ERE-E1b-CAT reporter, CMVβgal, and either pCMV₅-ERα, pCMV₅-ERβ_L or pCMV₅-ERβ_S expression plasmids and stimulated with EtOH, 1 nM estradiol, or 10μM forskolin + 100 μM IBMX. CAT measurements were made and corrected for CMVβgal activity, and values were standardized to the respective estradiol-stimulated activities. Transfections were performed in duplicate and values represent the mean and range for two experiments.

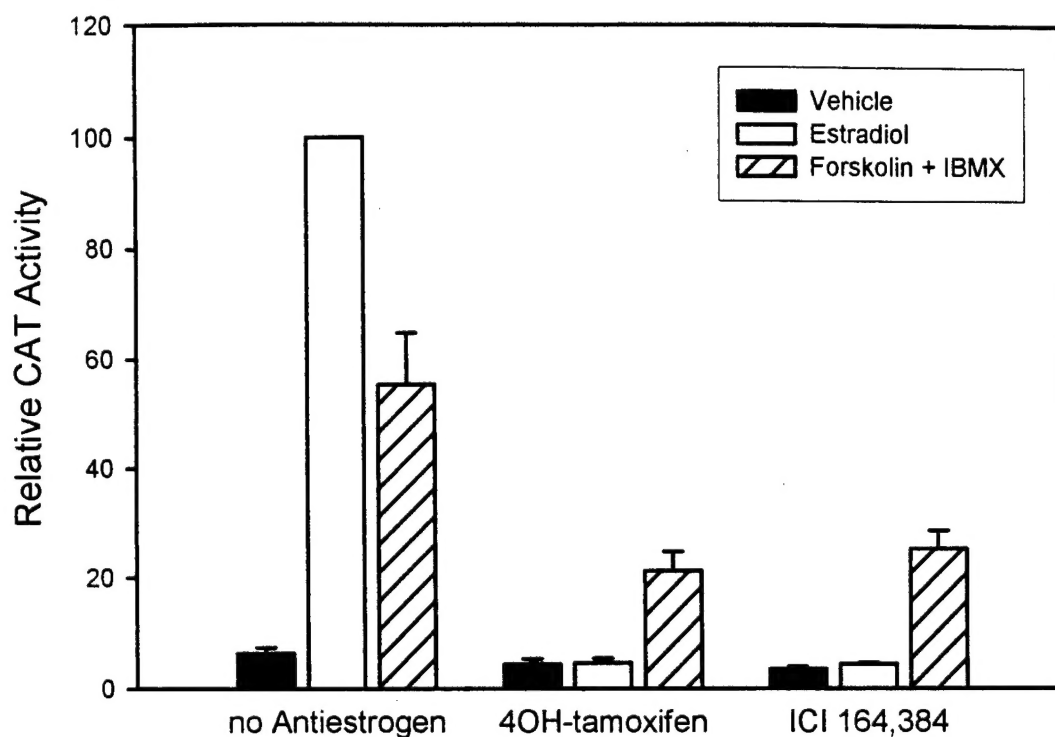


Figure 8: Activation of ER β _L Transcriptional Activity by Forskolin/IBMX and inhibition by Antiestrogens. HeLa cells were transfected with the 1 μ g ERE-E1b-CAT and 250 ng of pCMV₅-ER β _L expression plasmid. Luciferase measurements, normalized to cellular protein, are the results of stimulation with 1 nM estradiol or 10 μ M forskolin & 100 μ M IBMX in the absence or presence of 100 nM 4-hydroxytamoxifen or 100 nM ICI 164,384. Transfections were performed in duplicate and values are standardized relative to values obtained for cells treated with estradiol alone. Values represent the mean \pm SEM of five experiments.

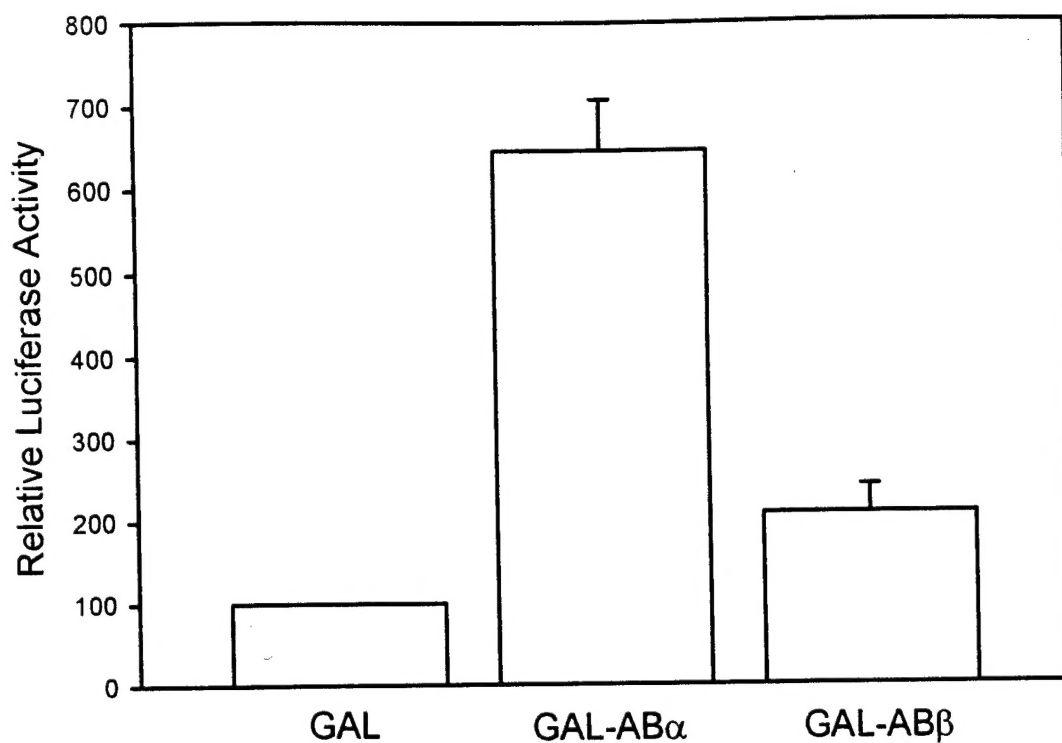


Figure 9: Transcriptional Activity of Gal-AB α and GAL-AB β Chimeric Proteins. HeLa cells were transfected with 100 ng of expression vectors for the GAL4 DNA binding domain alone (GAL), or the GAL4 DNA binding domain fused to either the A/B region of ER α (GAL-AB α) or ER β (GAL-AB β) with 1 μ g of pG5-Luc. Luciferase measurements were normalized to cellular protein. Transfections were performed in duplicate and values are standardized relative to values obtained for cells transfected with the expression vector for GAL4 DNA binding domain alone. Values represent the mean \pm SEM of four experiments.

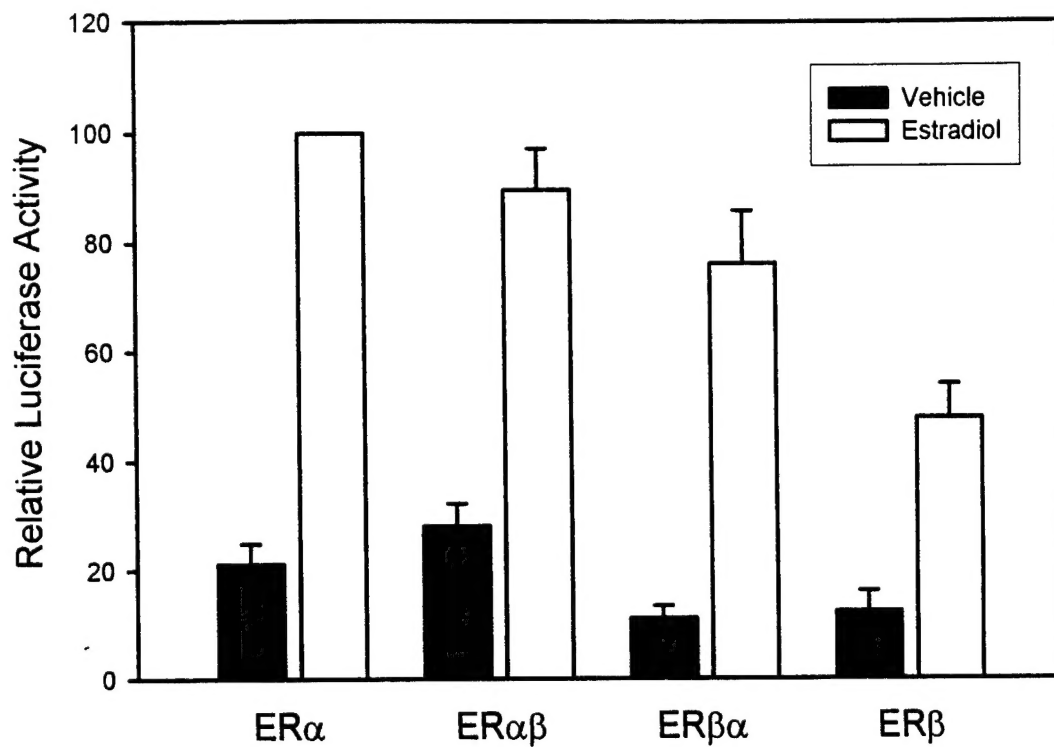


Figure 10: Transcriptional Activity of ER α and ER β Chimeras. HeLa cells were transfected with 100 ng of expression vectors for ER α , ER β or chimeras of the two receptors, ER $\alpha\beta$ or ER $\beta\alpha$ with 1 μ g of ERE-E1b-Luc. Luciferase measurements were normalized to cellular protein. Transfections were performed in duplicate and values are standardized relative to values obtained for cells transfected with the expression vector for ER α and treated with 1 nM estradiol. Values represent the mean \pm SEM of three experiments.

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ACTIVATION OF ESTROGEN RECEPTOR-ALPHA AND ESTROGEN RECEPTOR-BETA BY LIGAND-DEPENDENT AND LIGAND-INDEPENDENT PATHWAYS.

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The biological effects of estrogens are mediated via two intracellular proteins called the estrogen receptor- α (ER α) and estrogen receptor- β (ER β). In addition to the originally characterized short form of rat ER β which consists of 485 amino acids (ER β_S), a longer form of ER β has been described which also possesses an amino-terminal extension of 64 residues resulting in a protein with a predicted size of 549 amino acids (ER β_L). To analyze potential functional differences between ER α and the short and long forms of ER β , the ability of these receptors to transactivate target genes in transient transfection experiments in response to ligand-dependent and ligand-independent activation pathways was assessed in HeLa cells. The short form of ER β was created by deleting the first 399 nucleotides of the long form, rat ER β cDNA, and all receptor cDNAs were expressed from the same mammalian expression vector, pCMV₅. Western blot analysis indicated that the ER β_L cDNA encoded a protein of ~60 kDa, whereas the ER β_S cDNA produced an ~56 kDa protein. Although both isoforms of ER β were significantly less active than ER α in response to estradiol treatment, within the linear range of the assay ER β_S stimulated gene expression up to 1.5-fold higher than ER β_L . However, when very high levels of ER β_L and ER β_S expression vectors were introduced into cells, ER β_S -stimulated gene expression was only 70% of that induced by ER β_L . Hormone binding assays and Western blot analyses were performed to determine if differences in ER β_L - and ER β_S -dependent reporter gene activity were due to alterations in the relative expression of the two receptor isoforms. Hormone binding assays indicated up to 2-fold higher expression for ER β_S in comparison to ER β_L . Although this trend was confirmed by Western blots, this analysis indicated that when equal amounts of expression vector were introduced into HeLa cells, ER β_S expression was >10-fold more abundant than ER β_L . Dopamine-initiated intracellular signaling pathways have been demonstrated to activate ER α , and the ability of the synthetic D1 subtype dopamine receptor agonist, SKF-82958, to activate the long and short forms of ER β in a ligand-independent manner was assessed. Whereas SKF-82958 clearly activated ER α up to 3.8-fold in a dose-dependent manner, activation of either ER β_S or ER β_L was very weak (≤ 1.5 fold). Taken together, these results indicate that the relative expression of estrogen receptor isoforms must be taken into account when assessing their relative biological activity. Furthermore, ER α and ER β transactivate reporter gene expression differently when stimulated in a ligand-dependent or ligand-independent manner and this supports the hypothesis that these receptors exert unique biological activities *in vivo*. Supported by a grant from the Department of Defense Breast Cancer Research Program (DAMD17-98-1-8282).